Molecular genetic analysis for the A^{e1} and A^{3} alleles

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BACKGROUND: In addition to the common ABO phenotypes, numerous phenotypes with a weak expression of the A or B antigens on RBCs have been found. This study describes the molecular genetic analysis of the A_{el} and the A_3 phenotypes.

STUDY DESIGN AND METHODS: The seven-exon regions of the *ABO* genes of A_{el} and A_3B individuals were amplified by PCR and cloned, and the sequences of the exons and their adjacent splice sites were analyzed. Samples from 30 randomly selected A_1 individuals were also assessed.

RESULTS: The *A* gene with wild-type coding sequence was demonstrated in the A_{el} propositus, but all the six unrelated Taiwanese people with the A_{el} or $A_{el}B$ phenotype were shown to possess an *A* allele with the G→A mutation at the +5 position of intron 6 (IVS6+5G→A). RT-PCR analysis showed that the complete *A* transcript structure was absent in the A_{el} RNA samples. The A_3B individual possessed an *A* gene with an 838C→T missense mutation.

CONCLUSION: The results suggest an association of the A^{el*}*IVS6+5G→A* allele with the A_{el} phenotype in Taiwanese people. The mechanism defining how the A^{el*}*IVS6+5G→A* allele leads to the A_{el} phenotype awaits elucidation.

The ABO blood group system is without doubt the most important blood group system in transfusion medicine. In addition to the common ABO phenotypes, A_1, A_2, B, A_1B, A_2B, and O, numerous phenotypes with a weak expression of the A or B antigens on RBCs have been found. Each of these subgroups, such as A_{el}, A_3, A_x, cis AB, B_3, B_{el}, B_x, and B(A), and so forth, has their own defined serologic characteristics (reviewed in Watkins,[1] Issitt and Anstee,[2] and Daniels[3]). The molecular genetic basis of the ABO system was elucidated by Yamamoto et al.[4-6] in 1990, who first characterized the respective nucleotide sequences of three major alleles, A^1, *B,* and *O*, of the *ABO* locus. The α-1,3-*N*-acetylgalactosaminyltransferase (A transferase) encoded from the *A* allele catalyzes the transfer of GalNAc from the donor substrate UDP-GalNAc to the H structure (Fucα1-2Galβ1-R) to give the A determinant; the *B* allele encodes an α-1,3-galactosyltransferase (B transferase), which is responsible for the formation of the B determinant by the transfer of Gal from the UDP-Gal donor to the

ABBREVIATIONS: A transferase = α-1,3-*N*-acetylgalactosaminyltransferase; B transferase = α-1,3-galactosyltransferase; IVS = intervening sequence.

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same H precursor. Most of the *O* cDNA possesses a deletion at Nucleotide 261, which leads to reading frameshift and thus this allele fails to encode a complete transferase product. The *A*, *B*, and *O* cDNA structures are highly homologous. The primary amino acid sequences of the A and B transferases differ in only four residues, and it has been shown that the disparity of these four residues is responsible for the different donor substrate specificities between the two transferases.[7-10] The *ABO* gene was found to consist of at least seven exons,[11,12] which span approximately 19.5 kb of genomic DNA. Recently, it was found that, in addition to the known complete transcript structure, the *ABO* gene expresses another variant form, which contains a different starting exon 1 region through utilization of an alternative promoter.[13]

After the elucidation of the three major *ABO* alleles at the nucleotide level, several genotypes of the *A* and *B* suballeles responsible for the formation of subgroups were identified (reviewed in Chester and Olsson[14] and Yip[15]). It has been shown that a subgroup phenotype can result from a variety of molecular changes in the *ABO* allele. For example, four different suballeles, *A*1061Cdel*, *A*1054T*, *A*1054G*, and an *A-B* hybrid with 829 A suballeles, have been demonstrated to correlate with the A_2 phenotype[15,16] and two different nucleotide substitutions, 703A→G and 700C→G, in the *B* gene have been linked with the B(A) phenotype.[17,18] Most of these identified minor alleles have a mutation in the gene's coding sequence, and most of the mutations are single-nucleotide substitutions leading to amino acid alteration. Splice site mutations in the *A* and *B* alleles have also been correlated with subgroups. The *A* allele of the individuals with the A_{finn} phenotype was found to possess an A→G mutation at the +4 nucleotide of intron 6.[19] Moreover 13 of 14 Taiwanese people with the B_3 phenotype were shown to have a *B* allele with a G→A mutation at the +5 nucleotide of intron 3 (intervening sequence [IVS]3+5G→A) recently.[20] The IVS3+5G→A splice donor site mutation leads to the skipping of the exon 3 region of the *B* transcript during mRNA processing.

This study carries out a molecular genetic analysis of six samples from unrelated Taiwanese persons with the A_{el} phenotype (five with the A_{el} phenotype and one with the $A_{el}B$ phenotype) and one sample from a Taiwanese person with the A_3B phenotype. The A_{el} RBCs are not agglutinated by anti-A or anti-A, B, but the A antigen on the cells can be demonstrated after adsorption and elution. This phenotype was found to express in approximately 1 in 80,000 Taiwanese people.[21] The A_3 phenotype is characterized by mixed-field hemagglutination of RBCs with anti-A or anti-A, B. The A_3 phenotype is the least rare of the A subgroup in many populations; nevertheless, it was found to be much rarer than A_{el} in Taiwanese people.[21] Mutations in the *A* allele have been reported for the A_{el} and A_3 phenotypes in other ethnic groups;[22-25] however, different molecular bases were identified in our A_{el} and A_3 cases.

MATERIALS AND METHODS

Sequence analysis of the *ABO* gene

Genomic DNAs of 5 unrelated individuals with the A_{el} phenotype, 1 individual with the $A_{el}B$ phenotype, 1 individual with the A_3 phenotype, and 30 randomly selected individuals with the common A_1 phenotype were prepared from their peripheral blood cells with a DNA purification system (QIAamp DNA blood mini kit, Qiagen GmbH, Hilden, Germany). All of the assessed individuals belong to the Taiwanese populations, and informed consent was obtained from all participants. The seven-exon regions of the *ABO* genes were divided into four segments and amplified by PCR with specific primer sets as described previously.[18] The PCR products were cloned into the pCRII-TOPO vectors by a cloning kit (TOPO TA, Invitrogen, Groningen, the Netherlands). DNA sequences were determined with a sequencing kit (BigDye Terminator cycle sequencing kit, Applied Biosystems, Foster City, CA). Multiple clones from two batches of PCR products were sequenced to separate any PCR-induced errors from actual sequence polymorphisms.

Analysis of the *ABO* transcript structure

RT-PCR was employed to analyze the transcript structure of the *ABO* gene. Total RNA samples from three individuals with the A_{el} phenotype and from two individuals with the common A_1 phenotype were prepared from the buffy coats of peripheral blood cells with the QIAamp RNA blood mini kit (Qiagen). The first-strand cDNA samples were primed by oligo(dT) primer and synthesized by reverse transcriptase (ThermoScript, Invitrogen). Two rounds of PCR amplification then followed.

Two different sets of PCR primers were used for these samples. Two of the A_{el} samples (A_{el}-1 and A_{el}-2) and one A_1 sample (A_1-a) were amplified with the primer sets, and reaction conditions are described as follows. The first PCR procedure was performed with forward (TTGCG GACGCTGGCCGGAAAACCAAA, nucleotides 13-38 of *ABO* cDNA, spanning the exon 1-2 junction, codon for the initiation methionine as nucleotides 1-3) and reverse (GACGGGGCCTAGGCTTCAGTTACTCACAAC, antisense sequence, 99 nucleotides downstream from the stop codon in exon 7) primers. Products from the first PCR cycle were amplified by nested forward and reverse primers with the sequences CAAAATGCCACGCACTTC GACCTATGATCC (nucleotides 35-64, locating exon 2) and GTTCTTGGGCACCGCAGTGAACCTCAGCTT (complementary to nucleotides 1012-1041, in exon 7). The PCR reactions were performed in a final volume of 25 µL of PCR buffer containing 0.2 m*M* dNTP, 0.5 U of *Taq* polymerase, and 15 pmol of each forward and reverse primer. The PCR program used was 5 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 1 minute at 72°C.

The third A_{el} sample (A_{el}-3) and another A_1 sample (A_1-b) were amplified with primer sets made up of the same forward primers but with different reverse primers to those for the A_{el}-1, A_{el}-2, and A_1-a samples. The reaction conditions are described in a previous paper.[20] The sequences of the reverse primers employed for the A_1-b and A_{el}-3 samples were TGTCCACGTCCACGCACAC CAGGTAATCCA (complementary to nucleotides 611-640, in exon 7, for the first PCR) and CGCTCGCAGAAGT CACTGATC, complementary to nucleotides 573-593, locating exon 7, for the nested PCR.

The nested PCR products were analyzed by 2.0 percent agarose gel electrophoresis, and each amplified fragment was eluted from the agarose gel and sequenced to determine the structure.

RESULTS

Serology

Cases of A_{el} phenotype were discovered as a result of discrepancies between forward and reverse typing in routine ABO grouping by standard hemagglutination test. The RBCs of A_{el} phenotype were not agglutinated by polyclonal and monoclonal anti-A and anti-A, B reagents at room temperature. Adsorption and elution tests performed by testing A_{el} RBCs against polyclonal anti-A (Organon Teknika Corporation, Durham, NC) produced elutes reacting moderately with A_1 cells. The sera contained anti-B activity. The saliva contained only H substance, as determined by a salivary ABH inhibition test.

The RBCs of A_3 subgroup showed 1+ to 2+ mixed-field hemagglutination with monoclonal anti-A reagent and 2+ to 3+ mixed-field reaction with monoclonal anti-A, B reagent (Gamma Biologicals, Inc., Houston, TX). The serum sample contained anti-A activities but no anti-B activities. The saliva sample contained normal amounts of A and H substances.

A gene with the wild-type coding sequence but a point mutation at the splice donor site of intron 6 identified in the A_{el} propositus

The seven coding regions of the *ABO* gene of an individual with the A_{el} phenotype were PCR-amplified and cloned, and the sequences of the exons and the adjacent splice acceptor and splice donor sites were inspected. The results demonstrated that the individual harbored an A^1 and an O^1 gene with the respective wild-type coding sequences. However, the clones bearing the fragment encompassing the region from exon 6 to exon 7 amplified from the A^1 allele possessed a G→A substitution at the +5 nucleotide of intron 6 (IVS6+5G→A); the other clones bearing the exon 6-exon 7 fragment amplified from the O^1 allele had the wild-type G nucleotide at that position. Direct sequencing of the PCR product also showed the heterozygous state for the G and A nucleotides at that position (Fig. 1, right). No other nucleotide change was observed in the other splice donor and splice acceptor sites. Direct sequencing of the PCR product amplified from an individual with the common A_1 phenotype did not show the G to A change at the +5 position of intron 6 (Fig. 1, left).

The IVS6+5G→A mutation is present in the six unrelated cases with the A_{el} or $A_{el}B$ phenotypes, but is rare in the general Group A population

The exon 6-intron 6 junction of the *ABO* gene of the other four A_{el} individuals and one $A_{el}B$ individual were analyzed by PCR amplification and direct sequencing. All of the five samples showed a heterozygous state for the G and A nucleotides at the +5 position of intron 6 as demonstrated in the A_{el} propositus. The exon 6-exon 7 region of the *ABO* gene of the $A_{el}B$ case was further PCR-amplified, cloned, and sequence-analyzed, and the results showed that the IVS6+5G→A mutation was present in the *A* allele, not the *B* allele, of the individual. Thirty randomly selected individuals with the common A_1 phenotype were further analyzed, and none of the samples demonstrated a G to A change at that position.

These results indicate that all of the six unrelated A_{el} or $A_{el}B$ cases possess the *A* gene with the IVS6+5G→A mutation while the mutation is not detected in the general Group A population, suggesting an association of the A^{el}*IVS6+5G→A* allele with the A_{el} phenotype in Taiwanese populations. The IVS6+5G→A mutation leads to a change of the conserved splice donor site sequence from GTAAGT to GTAAAT (Fig. 2).

Complete *A* transcript was absent in the RNA samples from the A_{el} individuals with the A^{el}*IVS6+5G→A* allele

As the nucleotide substitution of IVS6+5G→A in the *A* gene changes the consensus sequence of a splice donor site ($GT^A/_GAGT$),[26,27] the transcript structures encoded from the *A* allele with the splice site mutation were inspected by RT-PCR. cDNA samples were synthesized from total RNA samples prepared from peripheral blood cells of three A_{el} individuals with the IVS6+5G→A mutation and two individuals with the common A_1 phenotype, and PCR amplifications were performed. One major fragment was obtained for each of the RNA sample from the common A_1 individual (Fig. 3A, lane A_1-a; Fig. 3B, lane A_1-b). Direct sequencing of these products revealed that the fragments were composed of the complete A^1 cDNA structure of the exon 2 through exon 7 regions. As different primer sets were used (see Materials and Methods and the legend to Fig. 3), the final products of complete A^1 cDNA

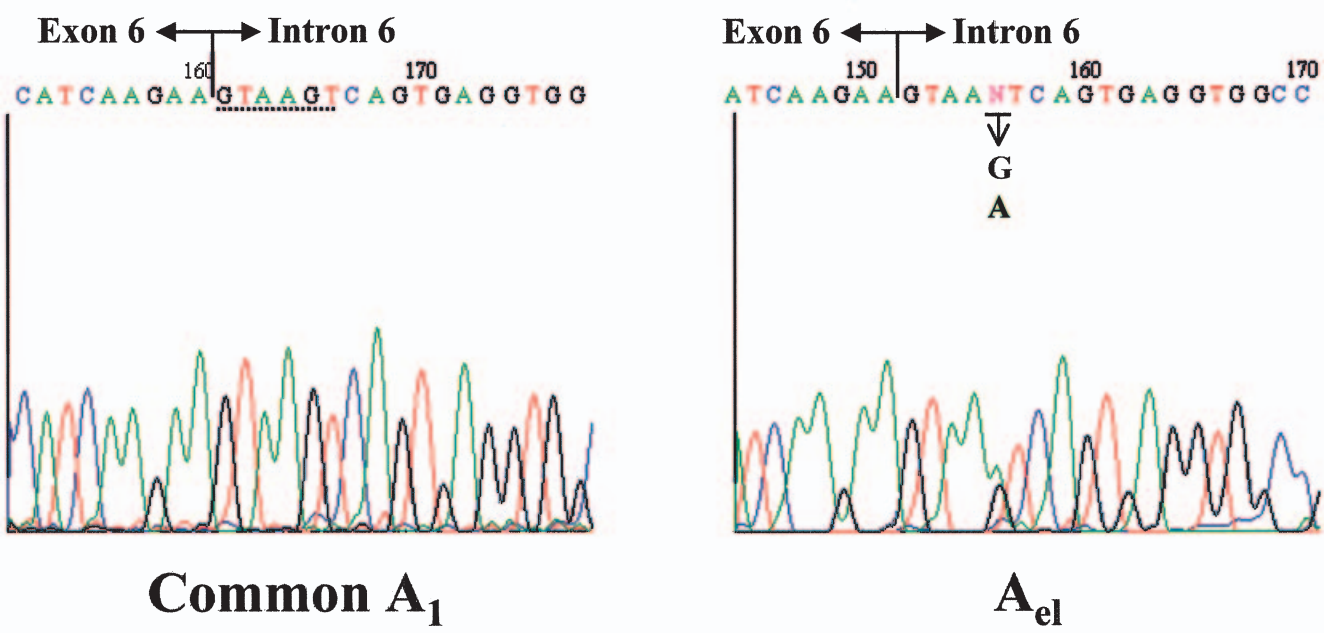

Fig. 1. Sequencing results of the exon 6-intron 6 junction of the *ABO* genes of a common Group A_1 individual and the A_{el} propositus. Genomic DNA samples were purified from an A_1 individual and the A_{el} propositus. The regions from exon 6 to exon 7 of the *ABO* genes were amplified by PCR, and the sequences were analyzed by direct sequencing. The sequencing results of the exon 6-intron 6 junctions of the common Group A_1 (left) and the Group A_{el} (right) samples are shown. In the A_{el} sample, the heterozygous state of the G and A nucleotides (underlined) at the +5 position of intron 6 is demonstrated. A dashed line indicates the conserved sequence of the splice donor site.

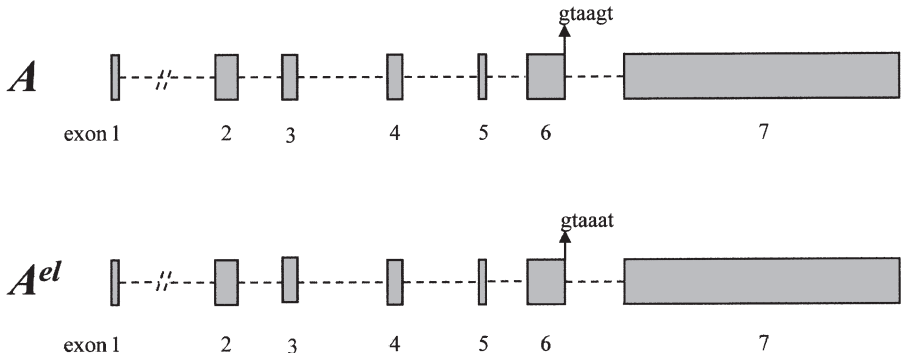

Fig. 2. A diagrammatic representation of the molecular basis for the A^{el} allele showing the IVS6+5G→A splice donor site mutation.

structures obtained from the A_1-a and A_1-b samples have different sizes of 1007 and 559 bp, respectively. However, the three RNA samples from the A_{el} individuals all failed to give a detectable complete *ABO* cDNA fragment, only yielding different alternatively spliced products (Fig. 3A, lanes A_{el}-1 and A_{el}-2; and Fig. 3B, lane A_{el}-3). Each of the A_{el} samples gave one major product of smaller size. Sequence analyses showed that these fragments were made up of the *ABO* cDNA with skipping of different exon regions or with retention of a segment of intron sequence. Not one of them had the same structure. The structures of these alternatively spliced products are described in detail in the legend to Fig. 3.

Although these A_{el} individuals possess an O^1 allele, the complete transcript encoded from the O^1 allele was not detected in the RT-PCR analyses. This phenomenon has been observed previously[20,28] and is believed to result from the decreased stability of the *O* allele transcript.

The RNA samples from the A_{el} individuals with the A^{el}*IVS6+5G→A* allele were all shown to be devoid of the complete *A* transcript structure, suggesting that the IVS6+5G→A mutation in the *A* gene results in a failure to produce a complete *A* transcript in these A_{el} RNA samples. However, a consistent alternative-spliced pattern for the *A* transcript in these samples was not demonstrated by RT-PCR analysis.

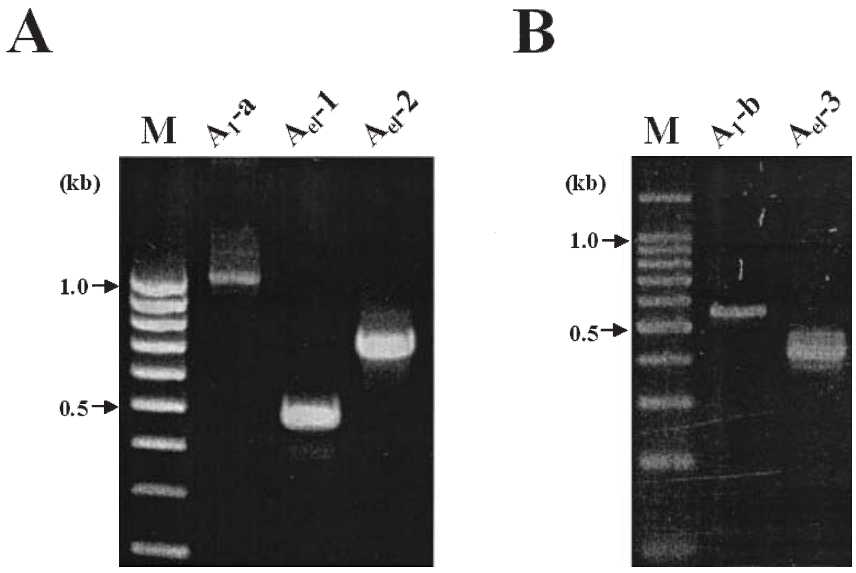

Fig. 3. *ABO* transcript structures of the common A_1 individuals and the A_{el} individuals with the IVS6+5G→A mutation analyzed by RT-PCR. The samples A_1-a, A_{el}-1, and A_{el}-2 (A) and samples A_1-b and A_{el}-3 (B) were amplified by two different PCR primer sets, which amplified the *ABO* cDNA regions encompassing the coding Nucleotides 35-1041 and 35-593, respectively (the full *A* and *B* cDNA samples comprise 1065 coding nucleotides and the codon for the initiation methionine are numbered as nucleotides 1-3), as described under Materials and Methods. The RT-PCR products were analyzed by 2.0 percent agarose gel electrophoresis. Lanes M, the molecular mass standards of the 100-bp ladder. The major fragment obtained from sample A_1-a (1007 bp) was demonstrated by sequence analysis to be composed of the complete A^1 cDNA structure of the exon 2 through exon 7 regions, whereas the major product amplified from sample A_{el}-1 was made up of the *ABO* cDNA structure with deletion of the coding nucleotides 99-750 (exon 3-6 and part of exon 7) but with retention of a segment from the intron 2 region (the 541th-677th nucleotides of the 724-bp intron 2 sequence, referring to the sequence deposited in GenBank with Accession No. AC000397) and yielded a PCR product of 498 bp. The major fragment shown in lane A_{el}-2 was made up of the *ABO* cDNA structure with skipping of the exon 3-6 region (coding nucleotides 99-374), yielding a product of 731 bp. The major fragment obtained from samples A_1-b was also demonstrated to be composed of the complete A^1 exons 2-7 cDNA structure and was 559 bp in size, whereas the RNA sample of A_{el}-3 gave a major product with a similar structure but with the exon 6 region (coding nucleotides 240-374) skipped, producing a 424-bp product. A minor product with a slightly larger mass than the 424-bp product was observed from the A_{el}-3 sample; however, we were unable to obtain a structure for this product by sequencing.

The A_3B individual possesses the *A* gene with an 838C→T missense mutation

The sequences of the seven exons and the adjacent splice acceptor and splice donor sites of the *ABO* gene of the A_3B individual were inspected after PCR amplification and cloning. This individual was shown to harbor a *B* gene with a wild-type coding sequence and an *A* gene containing a nucleotide change of 838C→T (translation initiation codon of *ABO* cDNA as nucleotides 1-3), which predicts an amino acid alteration of Leu280Phe in the encoded A transferase (Fig. 4).

The *ABO* exon 7 regions of 30 randomly selected Group A_1 individuals were PCR-amplified and sequence-analyzed through direct sequencing, and none of the A_1 individuals demonstrated the 838C→T mutation.

DISCUSSION

In this study, the molecular changes IVS6+5G→A and 838C→T, in the *A* gene, were identified in Taiwanese people with the A_{el} and A_3 phenotype, respectively. All of the six unrelated cases with the A_{el} or $A_{el}B$ phenotype were demonstrated to possess the IVS6+5G→A splice donor site mutation, suggesting the association of the A^{el}*IVS6+5G→A* allele with the A_{el} phenotype in Taiwanese populations. Previously the respective splice site mutation in the *A* and *B* alleles were shown to link with the A_{finn} and B_3 phenotypes, respectively.[19,20] The result reported in this article is the third case that represents an association of a splice junction mutation in the *ABO* gene with an ABO subgroup.

The highly conserved sequences at the splice donor and splice acceptor sites of the vertebrate and the primate genes have been comprehensively surveyed, and their conservation frequencies have been calculated.[26,27] At the splice donor sites, the G nucleotide at the +5 position has a high conservation frequency of about 84 percent, which is only slightly lower than those of the most conserved +1G and +2T nucleotides (both show 100% conservation at splice donor sites). Three different consequences of aberrant mRNA splicing owing to splice junction mutations have been observed: exon skipping, utilization of a cryptic splice site, and intron retention.[29,30] Among these consequences, exon skipping is most frequently observed.

The association of the B^3*IVS3+5G→A* allele with the B_3 phenotype in Taiwanese people was reported previously. It was further demonstrated that the complete *B* transcript, from exon 1 through to exon 7, was absent and the transcripts which skipped exon 3 were produced from the *B* gene with the IVS3+5G→A mutation instead.[20] It shows that the IVS3+5G→A mutation in the *B* gene

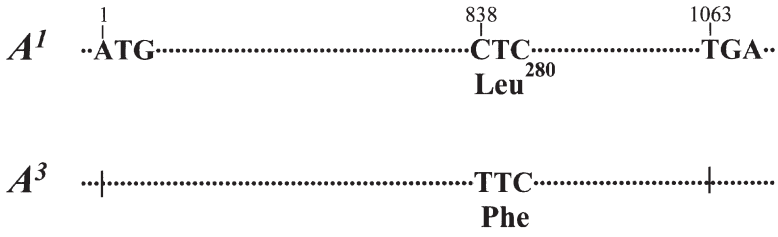

Fig. 4. The difference in nucleotide and predicted amino acid between A^1 and A^3 cDNAs. The A^3 cDNA reported in this article contains a C to T nucleotide substitution at position 838, and the nucleotide change predicts an amino acid alteration of Leu to Phe at residue 280.

destroys the consensus of the splice donor site and this leads to the skipping of the exon 3 region during mRNA splicing processes. The B^3 transcript without exon 3 predicts a B transferase product that lacks 19 amino acids in the N-terminal segment.

The RNA samples from the A_{el} individuals with the *A^{el}*IVS6+5G→A* allele were also shown to be devoid of the complete *A* transcript structure; however, a consistent alternative-spliced pattern was not demonstrated in the transcripts generated from the *A^{el}*IVS6+5G→A* allele. Splicing patterns of the *ABO* transcripts have been shown to be very complicated[6,11,13] and different alternative-spliced *ABO* transcripts have been obtained in different individuals with a common ABO phenotype with RT-PCR analysis in our laboratory. The three A_{el} individuals all possess, in addition to the A^{el} allele, an O^1 allele at their *ABO* loci. As all the major alternatively spliced transcript structures demonstrated in the A_{el} RNA samples did not contain the exon 6 region, in which the only nucleotide position (261G) that can distinguish the A^1 and O^1 alleles resides, we cannot confirm whether these alternative-spliced products were generated from the A^{e1} allele or from the O^1 allele. However, a full *A* or *B* cDNA structure can always be obtained from common A, B, or AB RNA samples with these RT-PCR systems in our laboratory, but the three A_{el} RNA samples analyzed all failed to demonstrate the complete *ABO* cDNA structure; thus, the results obtained from the RT-PCR analyses strongly supports the idea that the IVS6+5G→A mutation in the *A* gene results in a failure to produce a complete *A* transcript.

The +5G nucleotide is not 100 percent conserved at the splice donor site, and although most of the genes with the +5 position splice junction mutation showed 100 percent of exon skipping, some of the genes with the +5 position mutation produced, in addition to the major aberrant-spliced products, a minor portion of the normal transcripts.[29] Thus, we should not totally eliminate the possibility that trace amount of the complete *A* transcript, which had escaped aberrant splicing led by the IVS6+5G→A mutation and was not detectable by our RT-PCR analysis, did exist in the A_{el} RNAs. The presence of trace amount of normal A transferase in A_{el} may be a possible explanation for the presence of trace amount of A antigen on A_{el} RBCs. However, we doubt that the complete *A* transcript is present in our A_{el} samples as the RT-PCR (with nested PCR) analysis was sensitive and all three of the A_{el} RNA samples tested failed to demonstrate a complete *A* transcript structure even after large amounts of the alternatively spliced products had been obtained. Thus, the mechanism for the molecular basis of the A^{el} genotype, the IVS6+5G→A splice junction mutation, leading to the formation of the A_{el} phenotype still needs to be established.

It is of interest to note that the *A* allele possessing the A→G mutation at +4 position of intron 6 was demonstrated in four unrelated individuals with the A_{finn} phenotype.[19] This mutation changes the conserved splice donor site sequence from GTAAGT to GTAGGT of intron 6 of the *A* allele, while that identified in our A_{el} cases changes the conserved sequence to GTAAAT.

Two different molecular changes, 804insG and 646T→A in the *A* allele, have been reported to be responsible for the A_{el} phenotype formerly.[22,23] As for the A_3 phenotype, two different molecular changes, 871G→A and 829G→A with 1061delC, in the *A* allele have been reported previously.[24,25] Our A_3 case possesses the *A* allele with an 838C→T missense mutation. These studies demonstrate the genetic heterogeneity of these subgroups. Although the molecular bases for the A^3 allele (as well as several for the B^3 allele) have been revealed, the mechanism responsible for the formation of the special mixed-field hemagglutination of the A_3 (and B_3) RBCs still awaits elucidation.

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